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Abstract: The ecological relevance and factors shaping dynamics of Limnohabitans sp. have been largely studied by fluorescence in situ hybridization with a 16S rRNA probe targeting the R-BT group (lineages LimBCDE), but not lineage LimA. Consequently, ecology and distribution of LimA remained unknown. We developed a double hybridization strategy using a novel 23S rRNA probe specifically targeting LimA and LimE that in combination with the existing R-BT probe can discriminate LimA populations. This technique was applied for more than 1000 samples from 46 freshwater sites including long-term data sets from oligo-mesotrophic Lake Zurich, CH and meso-eutrophic Římov reservoir, CZ. LimA was ubiquitously distributed and highly abundant. Observed ecological preferences of LimA in Lake Zurich were in general similar to already reported for Limnohabitans with highest numbers in surface waters during growing seasons. Three times higher densities of LimA were detected in Římov reservoir, where they were significantly more abundant at the riverine zone especially after flood events that introduced fresh terrestrial DOM (dissolved organic matter). Moreover, statistical analyses of biological and physicochemical parameters obtained from small dynamic water bodies confirmed a correspondence between LimA and allochthonous DOM, in opposite to R-BT that was more related to algal primary production.

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Distribution and ecological preferences of the freshwater lineage LimA (genus *Limnohabitans)* revealed by a new double hybridisation approach

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Running head: Distribution of *Limnohabitans* lineage LimA

Originality-Significance Statement:

The family Comamonadaceae (Betaproteobacteria) displays restricted phylogenetic resolution of the 16S rRNA gene, commonly used for *in situ* detection of bacterial populations. In this study we overcome this limitation by using 23S rRNA genes from a large isolate collection for the design of a catalysed reporter deposition fluorescence *in situ* hybridisation (CARD-FISH) probe specifically targeting LimA and LimE lineages of the genus *Limnohabitans*. This genus is reported as ubiquitous and very abundant in freshwater environments. However, LimA lineage was known until now only from studies on isolates, while information about ecological distribution of this group was not available. Using a double hybridisation approach with newly designed LimAE and well known R-BT probes, we were able to provide first quantitative insight into ecology of LimA based on more than 1000 analysed samples from almost 50 ecosystems and report their environmental preferences partly contrasting to other *Limnohabitans* groups.

Summary

The ecological relevance and factors shaping dynamics of *Limnohabitans* sp. have been largely studied by fluorescence *in situ* hybridisation (CARD-FISH) with a 16S rRNA probe targeting the R-BT group (lineages LimBCDE), but not lineage LimA. Consequently, ecology and distribution of LimA remained unknown. We developed a double hybridisation strategy using a novel 23S rRNA probe specifically targeting LimA and LimE that in combination with the existing R-BT probe can discriminate LimA populations. This technique was applied for more than 1000 samples from 46 freshwater sites including long-term data sets from oligomesotrophic Lake Zurich, CH and mesoeutrophic Římov reservoir, CZ.

LimA was ubiquitously distributed and highly abundant. Observed ecological preferences of LimA in Lake Zurich were in general similar to already reported for *Limnohabitans* with highest numbers in surface waters during growing seasons. Three times higher densities of LimA were detected in Římov reservoir, where they were significantly more abundant at the riverine zone especially after flood events that introduced fresh terrestrial DOM (dissolved organic matter). Moreover, statistical analyses of biological and physicochemical parameters obtained from small dynamic water bodies confirmed a correspondence between LimA and allochthonous DOM, in opposite to R-BT that was more related to algal primary production.

Introduction

Betaproteobacteria are known as one of the dominant constituents of the freshwater bacterioplankton (Lindstrom et al., 2005; Barberan and Casamayor, 2010; Newton et al., 2011). Despite a high diversification among this group, only a few phylogenetically narrow lineages are of high ecological importance in inland waters (Zwart et al., 2002; Newton et al., 2011). The genera *Limnohabitans* (Hahn et al., 2010a; Kasalicky et al., 2010) and *Polynucleobacter* (Hahn, 2003; Hahn et al., 2009) have been reported as two of the most abundant and ubiquitous members of Betaproteobacteria in freshwater environments (Lindstrom et al., 2005; Salcher et al., 2008; Šimek et al., 2010; Newton et al., 2011; Jezbera et al., 2012). *Polynucleobacter* was described to harbour lineages with contrasting ecological strategies (Wu and Hahn, 2006; Hahn et al., 2016a): the PnecC lineage (*P. asymbioticus, P. duraquae, P. yangtzensis, P. sinensis* (Hahn et al., 2009; Hahn et al., 2016b) is mostly associated with low pH and high concentrations of allochthonous (especially humic) compounds (Jezberova et al., 2010), while abundances of lineage PnecB (*P. acidiphobus*, Hahn et al., 2011) are positively related to pH and phytoplankton primary production (Hahn et al., 2011; Salcher et al., 2011b; Jezbera et al., 2012). *Limnohabitans* spp. on the other hand seem to be ecologically more uniform and display clear environmental preferences similar to PnecB (Šimek et al., 2010; Jezbera et al., 2012).

The genus *Limnohabitans* contains four described species, i.e., *L. parvus, L. planktonicus, L. australis* and *L. curvus* (Hahn et al., 2010b; Hahn et al., 2010a; Kasalicky et al., 2010) and was initially resolved in four lineages (LhabA1-4, Newton et al., 2011). The phylogenetic structure was subsequently revised and redefined in five lineages (LimA-E) after sequence analyses of 16S rDNA and intergenic spacers between 16S and 23S rRNA genes (ITS) of a large isolate collection and environmental clones (Kasalicky et al., 2013). The majority of existing environmental data on *Limnohabitans* results from catalyzed reporter deposition fluorescent *in situ* hybridisation (CARD-FISH) analyses with the widely used probe R-BT065 (Šimek et al., 2001), targeting lineages LimB, C, D and E. The R-BT group is characterized as (i) fast growing opportunistic or copiotrophic (Horňak et al., 2006; Šimek et al., 2006; Horňak et al., 2008), (ii) preferring phytoplankton-derived organic material (Perez

and Sommaruga, 2006; Allers et al., 2007; Perez and Sommaruga, 2007; Šimek et al., 2007; Šimek et al., 2008), (iii) possessing diversified metabolic abilities (Kasalicky et al., 2013; Salcher et al., 2013), and (iv) being highly vulnerable to flagellate grazing (Jezbera et al., 2005, 2006; Šimek et al., 2007; Šimek et al., 2014).

However, far less is known about the LimA lineage, as it is not targeted by probe R-BT065 (Šimek et al., 2001; Kasalicky et al., 2013). Precise characterization of the LimA lineage in environmental samples was so far hampered by the poor phylogenetic resolution of the 16S rRNA gene for all Comamonadaceae (Wen et al., 1999; Kasalicky et al., 2013), especially when dealing with short sequences from, e.g., amplicon NGS studies (Shabarova et al., 2014). Despite this, a high ecological relevance of LimA was already suggested by Jezbera and co-authors (2013) by using a qualitative method based on ITS sequences (Reverse Line Blot Hybridisation, RLBH). In their study, approx. 60% of 161 tested samples from 72 sites were hybridised with a RLBH-probe specific for the LimA lineage, along a wide pH gradient (from < 4.5 to >9; Jezbera et al., 2013). Metabolic profiling of LimA isolates suggested that *L. curvus* strain MWH-C50^T and *L. australis* strain MWH BRAZ-DAM-2D^T (Kasalicky et al., 2010; Kasalicky et al., 2013) utilize a large number of common substrates (11 of 37 tested) with a clear preference for carboxylic acids (5 of 11). Still, the lack of quantitative data on LimA from freshwater environments constitutes a severe gap in understanding of the ecology and niche distribution within the genus *Limnohabitans*.

The goal of this study was (i) to provide a method for *in situ* detection and quantification of LimA, (ii) to characterise ecological and physicochemical preferences of this lineage based on temporal and spatial distribution at different sites, (iii) to compare life strategies and ecological niches of LimA to related members of the genus *Limnohabitans* and other Betaproteobacteria.

Results

Phylogenetic analysis and probe design

Sequences of 23S rRNA genes of 59 *Limnohabitans* strains from the isolate

collections of V. Kasalicky and M.M. Salcher were used for phylogenetic analysis of the genus *Limnohabitans*. The maximum likelihood tree revealed two robust phylogenetic groups within the genus: LimAE and LimBC (Fig. 1) with within-group sequence identities of >98.7% and >97.8%, respectively. The difference between LimAE and LimBC ranged from 2.4% to 3.5% (average 2.9%), while the distance between *Limnohabitans* and related genera was on average 3.2%. Recently isolated strains formed five well separated groups in the tree, i.e. two within LimA represented by isolates from Jiřicka pond, CZ; two within LimC and one within LimB, represented by isolates from Lake Zurich (Fig. 1). A tree based on 16S rRNA gene sequences displayed relatively robust separation of LimC and LimB groups within the genus, but an unstable positioning of the LimAE cluster (Fig. S1).

The 23S rRNA probe LimAE-1435 (5'-TCC AAC AGT CTG CTG AGC TAA CC-3') designed for the monophyletic group LimAE has 100% group-coverage and zero outgroup-hits (two sequences affiliated with *Chromobacterium* [JTGE01000035, JWJN01000034] were targeted with one mismatch and 432 additional sequences with two mismatches, most of them could be addressed by using two competitors: 5'-TCC AAC AGT TGG CTG AGC TAA CC-3' and 5'-TCC AAC AGT CTG CTA ACC TAA CC-3'). Stringent conditions were achieved with 60% formamide in the hybridisation buffer and in silico and *in situ* tests on isolates showed that the double hybridisation with R-BT065 probe can discriminate between LimA, LimE and LimBCD (Fig. 2).

Abundance of LimA and LimE lineages in different aquatic habitats

Lake Zurich, CH

Lineage LimA was detected in all samples collected during a three-year monitoring of the whole water column of Lake Zurich (Table 1). Relative abundances of LimA were higher than quantification limit (0.2% of DAPI stained cells) in most cases (654 of 737 samples). Bacteria hybridised with probe R-BT065 were present in all samples and only in three cases exact quantification was not possible. However, double hybridised cells (corresponding to LimE) were either not present or were below the limit of quantification in 84.7% of samples. All three bacterial groups showed recurrent increases in absolute and relative abundances in the epilimnion during the growing season, however, with maxima at different time points (Fig. 3b, c, e, f). In 2012, for example, R-BT had a maximum in early spring (28 March: 6.5% of

158 DAPI; 2.7 ×10^5 cells ml^{-1}), LimE in late spring (10 May: 1.5%; 0.4×10^5 cells ml^{-1}) and
159 LimA in early autumn (11 September: 3.2%; 1.1×10^5 cells ml^{-1}). Redundancy
160 analysis (RDA) performed for epilimnetic samples and for the whole water column
161 (data not shown) gave very similar results, except for a higher significance of
162 turbidity and organic nitrogen concentration (Fig. 3g, Table S1). Environmental
163 variables accounted for 61% of total variation of LimA and R-BT abundances,
164 displaying positive correlation of both bacterial groups with temperature,
165 concentrations of oxygen, ammonium, dissolved organic carbon (DOC) and organic
166 nitrogen, turbidity, and chlorophyll *a* of all algal groups; and negative correlations
167 with concentrations of cyanobacterial (*P. rubescens*) chlorophyll *a*, phosphate and
168 nitrate concentrations, and sample depth (Fig. 3g, Table S1). According to
169 partitioning of variations, explanatory values of different chlorophyll a types and
170 phytoplankton biomass account for 12.3% and 2.6% of total variance of abundances
171 of R-BT and LimA, respectively. In opposite, physicochemical parameters (sampling
172 depth, conductivity, temperature, concentrations of nitrate, ammonium, phosphate,
173 dissolved phosphorus and DOC) showed a two times higher explanatory value
174 (26%) for variance of LimA abundances (Fig. 3h, Table S2).
175 **Římov reservoir, CZ**
176 Longitudinal monitoring of Římov reservoir in 2011-2013 included three stations:
177 River, Middle and Dam, and resulted in 168 samples. Relative abundances of LimA,
178 LimE and R-BT were above the limit of quantification in 155, 62 and all of them,
179 respectively (Table 1). LimE were not very abundant with a maximum on 1 October
180 2013 at the Dam (1.9%; 0.65×10^5 cells ml^{-1}). The samples collected from Dam and
181 Middle stations displayed similar proportions and numbers of LimA (1.9±1.8% and
182 1.8±2.3% or 0.5±0.4×10^5 and 0.6±0.7×10^5 cells ml^{-1}, respectively). In contrast,
183 significantly higher relative and absolute abundances of LimA ($P<0.05$) were
184 observed in riverine samples (River; 4.3±1.9%; 1.3±0.7×10^5 cells ml^{-1}, Fig. 4a). No
185 significant differences in absolute abundances of R-BT were detected for the three
186 stations (Dam: 2.4±1.3×10^5 cells ml^{-1}; Middle: 2.5±1.6×10^5 cells ml^{-1}; River:
187 2.6±2.2×10^5 cells ml^{-1}). However, relative abundances of R-BT were significantly
188 lower at the Middle than at the River, but not at the Dam (Dam: 6.3±3.2%; Middle:
189 5.7±3.4%; River: 8.0±4.4%, Fig. 4b). Three flood events with flow rates >17.8 m^3 s^{-1}
190 (average flow rate + 2 standard deviations) took place in 2012 and 2013 (Fig. 5a).
191 Relative but not absolute abundances of LimA were significantly higher within a

three-week period after these pronounced floodings than during antecedent low flow hydrological conditions ($P<0.01$, Fig. 5b). No significant effect of flood events was observed for R-BT abundances (data not shown). RDA analysis explained 45% of total data variance (Fig. 4c, Table S3). Significant positive correlations were observed between LimA and station River, season Spring, A370, as well as dissolved reactive silica (DRSi), phosphorus and nitrate concentrations; while they were negatively related to season Summer, oxygen concentrations and water temperature. Moreover, pH as well as cyanobacterial and total chlorophyll a values displayed negative correlations with both analysed bacterial groups. No clear pattern was detected for abundances of R-BT and variation partitioning showed only weak connections between phytoplankton associated characteristics and abundances of both *Limnohabitans* groups (<1% of variance). Physicochemical parameters explained 30% and 7% of variance of LimA and R-BT respectively.

Diverse freshwater habitats in Central Europe

LimA was detected in 42 and LimE in 29 out of 50 additionally analysed freshwater systems (lakes, ponds, pools, springs, peat bogs; Table 1, Tables S5-S6, Fig. S2). Highest relative abundances of LimA were observed in ponds and karst springs: forest pond Huťsky, CZ (18.6%), shallow high mountain pond Trog 1, AT (17.6%), Branna sand-pit pond, CZ (17.3%), Milandre karst spring, CH (17.3%), and a karst spring close to Lake Thun, CH (8.7%). LimA abundances were lower in lakes where they reached up to 6.1% of DAPI stained cells. Neither LimA nor LimE were detected in four peat bogs and in pools of a karst cave. Although most samples showed very low relative abundances of LimE (<3%), this lineage accounted for 9.2 - 14.3% of all cells in a depth profile from the high-mountain lake Gossenkollesee, AT, while LimA were virtually absent there (Fig. S2). R-BT abundances were highest in ponds (up to 28.8%) and lakes (up to 14.3%) and were not detected in springs, cave pools and peat bogs.

Canonical correspondence analysis (CCA) based on absolute abundances and a wide range of physicochemical parameters from 28 samples collected from 22 hydrological highly dynamic systems listed in Table S6 revealed a strong correspondence between LimA, the $SUVA_{254}$ index (proxy for aromaticity of DOC), as well as correspondence between R-BT, pH, conductivity and chlorophyll *a* and between PnecC and A250 (proxy for concentration of humic substances; Fig. 6, Table S4).

Cultures of algae, cyanobacteria and macrophytes

We detected LimA in one out of 10 tested samples from algal and cyanobacterial cultures (1.2% in *Dolichospermum* sp., Table S5). R-BT in contrast were present in 8 of these samples in relatively high percentages (up to 26.2%, Table 1). On the other hand, LimA - but not R-BT - were highly abundant in the surrounding water of cultured aquatic macrophytes *Utricularia* spp. (3.9 - 45.5%; 7.3×10^4 - 9.0×10^5 cells ml^{-1} and 0 – 1.7%; 0 - 4.0×10^4, respectively). LimE were not present in any of these samples.

Physiological profiling of strains affiliated with LimA and LimC

The five tested LimA strains showed limited physiological abilities and grew on only nine (isolate SP3) to 19 substrates (isolates Rim8 and *L. australis* MWH-BRAZ DAM-2D^T) from 95 tested. Representatives of LimC (*L. parvus* II-B4^T, *L. planktonicus* II-D5^T and Rim47) demonstrated a diversified metabolism and were able to growth on 41 (II-D5) to 87 (Rim47) substrates (Fig. 7a, Table S7). The number of compound classes utilized by LimA was limited to seven out of 14 (including amino sugars and dipeptides not depicted in Fig. 7a), while the LimC strains could metabolise all 14 classes. Principal component analysis (PCA) revealed a clear similarity in assimilation pattern of LimA strains, while LimC strains displayed a wide variation (Fig. 7b). The highest growth potential of all LimA was observed on carboxylic acids and the sugar acid dulcitol while LimC did not display any clear preferences (Table S7).

Discussion

Phylogeny of the genus Limnohabitans based on 23S rRNA gene sequences.

A clear phylogenetic separation of LimAE and LimBC was based on both remarkably high within-group sequence identities and differences between them. This allowed us to design a probe specific for LimAE, as well as to distinguish between all lineages of the genus, except for LimD due to a lack of available sequences. However, the resolution of the 23S rRNA gene was still not sufficient to provide a stable phylogeny for other members of Comamonadaceae (Fig. 1), already reported as problematic (Wen et al., 1999; Kasalicky et al., 2010; Kasalicky et al., 2013). Moreover, the

distance between LimA and LimE lineages based on 23S rRNA gene sequences was much smaller than observed for 16S rRNA gene analysis, especially in combination with ITS (<1.3% 23S, <3% 16S, <13.3% 16S+ITS; Kasalicky et al., 2013). A similar situation was observed between LimB and LimC (<2.2% 23S, <3% 16S, <16.7% 16S+ITS). It is therefore not surprising that 23S rRNA gene sequences did not allow us to design a probe specific exclusively for LimA or to differentiate between sub-lineages of LimC proposed in Kasalicky et al., 2013. Despite this, we were able to recognize some groups comprising novel isolates that were clearly separated from known strains by high bootstrap values in both 23S and 16S rRNA gene sequences based trees (Fig. 1, Fig. S1). Some of them can be considered as sub-lineages, e.g. group I, displaying the longest branch and harboring nine LimA strains isolated from Jiřicka pond in 2014 shortly after a pronounced flood event (Fig. 1, unpublished data).

Spatial and temporal distribution of LimA and other Betaproteobacteria in Lake Zurich and the Římov reservoir

Absolute abundances of LimA and R-BT in oligomesotrophic Lake Zurich showed a very similar seasonal and spatial distribution with maxima in the warm epilimnion in summer (Fig. 3c, f and g; Pearson correlation between groups $R=0.76$, $P<0.0001$), comparable to other heterotrophic bacterial populations such as LD12 Alphaproteobacteria or PnecB (Wu and Hahn, 2006; Salcher et al., 2011a). Moreover, all above mentioned bacterial groups were reported to avoid the cyanobacterium P. rubescens (Salcher et al., 2011b; Fig. 3g). However, the occurrence of the *Limnohabitans* groups appeared to be driven by different factors: R-BT was clearly associated with algal primary production as deduced from variation partitioning and a strong correlation between R-BT numbers and oxygen concentrations, different types of algal chlorophyll a, especially chlorophyll a associated with diatoms (Fig. 3a, c, g and h), as was already reported before (Salcher et al., 2011b; Eckert et al., 2012). In contrast, LimA showed only a slight correlation with chlorophyll a of green algae (Fig. 3d and f), but physicochemical parameters such as water temperature and concentrations of ammonium and DOC seem to be more important factors influencing LimA in Lake Zurich (Fig. 3g and h). The Římov reservoir, a freshwater system with higher trophic status, 5 times shorter retention time and consequentially higher allochthonous input than Lake Zurich,

harboured more than 3 times higher relative and absolute abundances of LimA (Table 1). We also observed a clear pattern along the reservoir with LimA being significantly more abundant at the River station than at the two downstream stations. A similar trend was also observed for PnecC in 2005 (Jezbera et al., 2012), where PnecC abundances were also correlated to concentrations of humic compounds and dissolved reactive phosphorus. Thus, LimA and PnecC abundances seem to be connected to allochthonous influence of the river. R-BT were distributed more or less equally along the reservoir and did not show any significant correlations with hydrological parameters, in agreement with a previous study (Jezbera et al., 2012), emphasizing the high metabolic and ecological diversification within this *Limnohabitans* group (Kasalicky et al., 2013). The observed negative correlation between total chlorophyll a values and other proxies of primary production (mainly associated with cyanobacteria in this period, Fig. 5) and abundances of both *Limnohabitans* groups in Řimov reservoir supports the previous observations of a mutually exclusive distribution of *Limnohabitans* and cyanobacteria (Fig. 3, Horňak et al., 2008; Salcher et al., 2011b).

Distribution of LimA in other habitats

Higher abundances of LimA were observed in small freshwater ponds and springs highly influenced by terrestrial systems than in large circum-neutral lakes (Table 1). In highly dynamic water bodies abundances of LimA showed a general positive correlation with PnecC and negative with R-BT and were associated more with allochthonous DOM such as humic acids than with autochthonous (Fig. 6, (Šimek et al., 2010; Jezbera et al., 2012). Remarkably, the $SUVA_{254}$ index (proxy for DOM aromaticity) displayed a strong correspondence to LimA abundances (Fig. 6). A link between floods and elevated $SUVA_{254}$ as an indicator for introduction of allochthonous DOM was shown for streams and a river-floodplain system (Hood et al., 2006; Sieczko and Peduzzi, 2014). Thus, we hypothesise direct or indirect connections between LimA and terrestrial environments, which can serve as potential source of this microbial group and/or provide suitable DOM during flooding events. The correspondence with $SUVA_{254}$ was less pronounced for PnecC, which is assumed to consume photo-degradation products of humic compounds (Watanabe et al., 2009; Jezberova et al., 2010; Hahn et al., 2012; Jezbera et al., 2012). R-BT in dynamic waterbodies showed again positive relationships with

variables related to algal primary production, i.e. chlorophyll a, and pH.
The effect of pH on the occurrence of LimA seems to be negligible, as they were detected in systems with pH values ranging from 5.8 to 8.9 (Table S6), which is in accordance with earlier findings (Jezbera et al., 2013), although we could not detect LimA in freshwater bodies with low pH such as peat bogs (pH 4-5, Table S3).
High numbers of LimA (up to 45.5% of DAPI stained cells) were found in cultures of different *Utricularia* spp. and in a sand pit pool overgrown by macrophytes (Table S5). This hints at a possible connection between LimA but not R-BT and higher plants as a part of the terrestrial ecosystem that might provide suitable organic substrates supporting their growth. However, almost no LimA cells were detected in algal cultures (Table S5), thus they do not seem to profit from algal exudates, similar to PnecC (Šimek et al., 2011; Hahn et al., 2012). R-BT as well as PnecB, on the other hand, are known to be connected to algal derived organic matter (Šimek et al., 2011).

Metabolic abilities of isolates

LimA strains displayed very limited and selective metabolic abilities especially in comparison to the tested LimC isolates. Among compound classes metabolized by LimA, highest percentages were displayed for carboxylic acids (36-50%), in agreement with previous studies (Kasalicky et al., 2010; Kasalicky et al., 2013), followed by carbohydrates (16-44%) and amino acids (0-26%, Fig. 7a). In freshwater habitats, these three low molecular weight compound groups might be of autochthonous origin, but are also known to be brought in high amounts by floods (Berggren et al., 2010). Thus, the observed correlation between LimA and $SUVA_{254}$ might not be necessarily related to the degradation of aromatic compounds, but rather indicates their connection to flood events. Interestingly, four out of five LimA strains displayed the highest growth potential on dulcitol (galactitol) – a sugar acid compound contained in red algae and some higher plants (Noiraud et al., 2001). LimC strains, on the contrary, showed a more equal distribution of metabolised substrate classes. This is in agreement with a high metabolic versatility observed for R-BT *in situ* (Salcher et al., 2013), and their tight linkage to organic nitrogen sources such as amino acids (Fig. 3g). *L. planktonicus* displayed the lowest metabolic diversification among the tested LimC isolates, which might be related to the specific lifestyle as a *Daphnia* sp. epibiont reported for this strain (Eckert and Pernthaler,

2014; Peerakietkhajorn et al., 2016).

Conclusions

This study presents the first comprehensive analysis of the occurrence and ecological relevance of the so far 'hidden' group LimA of the genus *Limnohabitans* by applying a novel hybridisation strategy. Our data revealed a wide distribution and high densities of LimA populations in freshwater environments and allowed us to draw first conclusions about LimA dynamics and potential factors shaping their populations. For example, LimA repetitively displayed higher abundances in surface waters during warm seasons, as well as mutually exclusive distribution with cyanobacteria, corresponding to preferences known for its sister group R-BT. In contrast to R-BT, LimA showed limited connections to algal primary production. Instead we observed high abundances of LimA in dynamic environments impacted by terrestrial DOM and higher plants exudates, often in combination with low densities of R-BT. Thus, the current description of ecological preferences within the genus *Limnohabitans* is similar to the dichotomy observed within *Polynucleobacter*, i.e. the niche separation between PnecC and PnecB. The environmental preferences of LimA seem to be more similar to PnecC than to R-BT, which in turn appears to be ecologically closer to PnecB. We also provide first evidence for a connection between LimA abundances and hydrological regime, i.e. flood events, a topic that deserves detailed further investigations to disentangle the ecological niches of LimA and PnecC.

Experimental Procedures

Phylogenetic analysis and probe design

The ARB software (Ludwig et al., 2004), SILVA database LSU_119 (Pruesse et al., 2007) and already existing oligonucleotides (Hunt et al., 2006) were used to design five primers targeting 23S rRNA genes of *Limnohabitans* spp. (Text S1). DNA of 59 *Limnohabitans* strains from the isolate collections of V. Kasalicky and M.M. Salcher was used for amplification and sequencing of 23S rRNA genes with the newly designed primers (Text S1). Sequences were assembled with Geneious R7 software (www.geneious.com), aligned with the SINA web aligner (www.arb.silva.de) and

merged with the SILVA database LSU_123. Thereafter, sequences of isolates and type strains of closely related genera were trimmed to approx. 2100 bp (503 to 2630 position in E. coli) and a maximum likelihood tree was constructed after additional alignment with MAFFT (Katoh et al., 2002) with FastTree2 (Price et al., 2010) using a GTR+CAT model and an estimation of the gamma parameter. Bootstrapping of 100 trees was performed with the seqboot program of the Phylip package (Retief, 1999) with *Comamonas testosteroni* serving as out-group. The probe LimAE-1435 and two competitors were designed for the monophyletic group LimAE in ARB and evaluated with the web tool mathFISH (Yilmaz et al., 2011). An equivalent tree based on 16S rRNA gene sequences was constructed using the same *Limnohabitans* strains and additional LimAE environmental clone sequences from Lake Zurich and Římov Reservoir. All sequences have been submitted to EMBL under accession numbers LT555974-LT556033 (23S rRNA) and LT717398-LT717424 (16S rRNA).

Study sites and sampling

We analysed four different sample sets to assess the distribution of LimA lineage in the context of available environmental parameters and co-occurrence with selected groups of Betaproteobacteria (R-BT, PnecC).

1. Lake Zurich (three-year monitoring of the water column)

Lake Zurich is an oligomesotrophic, prealpine, monomictic lake in Switzerland. This very well described freshwater ecosystem with intense monitoring since 1976 is particularly known because of changing dynamics of the toxic cyanobacterium *Planktothrix rubescens* (Posch et al., 2012). Sampling was conducted every two weeks including vertical profiles of physicochemical parameters using a YSI multiprobe (Yellow Springs Instruments, model 6600, Yellow Springs, OH, USA) and profiles of four phytoplankton groups (cyanobacteria, green algae, diatoms and cryptophytes) differentiated by different fluorescent spectra using a submersible fluorescence probe (FluoroProbe, TS-16-12, bbe Moldaenke GmbH, Schwentinental, Germany). Water samples for bacterial analyses were taken from 0, 5, 10, 20, 30, 40, 60, 80, 100m, and at the depth of chlorophyll a maximum. Samples for determination of concentrations of nitrogen, organic carbon and phosphorus were taken monthly. For more details see Salcher et al., 2015 and Table S8. Samples from three years (5 January 2010 to 18 December 2012; n=737) were used in this

study.

2. Římov reservoir (three-year monitoring of the longitudinal profile)

The canyon-shaped meso-eutrophic Římov reservoir is located in South Bohemia (Czech Republic) and serves as an important source of drinking water (Šimek et al., 2008). The samplings were conducted along its longitudinal axis in one to three weeks intervals during the ice-free period in the years 2011-2013 (n=168). Water samples were taken from 0.5 m depth from three sites of the reservoir: Dam, Middle and River (inflow) (Šimek et al., 2008). Concentrations of total and dissolved reactive phosphorus (TP, DRP) and nitrate were quantified as described previously (Rychtecky and Znachor, 2011). Dissolved oxygen concentrations and pH were measured *in situ* (WTW Oxi 330i; WTW, Weilheim, Germany). Chlorophyll *a* of cyanobacteria, green algae, diatoms and cryptophytes and fluorescence of "yellow substances" (excitation at 370 nm) were determined with a submersible fluorescence probe (FluoroProbe, TS-16-12, bbe Moldaenke GmbH, Schwentinental, Germany). Daily flow rate data of the reservoir inlet were obtained from the River Vltava Water Authority, State Enterprise.

3. Comparative survey of diverse freshwater habitats in Central Europe

Remaining samples (n=33, Table S6) from an extensive sampling campaign of various aquatic ecosystems in Czech Republic and Austria (Šimek et al., 2010) were used to quantify LimA and LimE. Data from 28 samples taken from hydrologically highly dynamic sites were selected for additional statistical analysis of abundances of LimA in comparison to R-BT and PnecC in context of a large number of physicochemical parameters (Šimek et al., 2010). Physicochemical parameters accompanying this dataset include several absorbance characteristics used as proxies for DOC composition: (i) $SUVA_{254}$ – a ratio of specific UV absorbance measured at 254 nm to DOC concentration (A254/DOC), corresponding to aromaticity of dissolved organic carbon; (ii) A250 – a specific UV absorbance measured at 250 nm, corresponding to concentrations of humic substances; (iii) A250/A365 – a ratio between specific absorbances measured at 250 and 365 nm, correlating with proportions of low molecular DOC (Šimek et al., 2010). All specific absorbances were measured with a Specord 210 dual-beam spectrophotometer (Analytik, Jena, Germany). In addition, water samples from 17 more aquatic environments from Switzerland, Austria and Czech Republic without corresponding physicochemical parameters were examined (n=65, Table S5, Fig. S2).

4. Non-axenic cultures of algae, cyanobacteria and macrophytes

Additional samples were gathered from non-axenic cultures of algae, cyanobacteria and macrophytes (n=13, Table S5).

Determination of bacterial abundances and CARD-FISH analyses

Water samples from different sites were fixed with formaldehyde or paraformaldehyde (final concentration 2%) within 2 hours after sampling. Bacterial abundances were determined after DAPI (4',6-diamidino-2-phenylindole) staining via epifluorescence microscopy or flow cytometry (Šimek et al., 2008; Šimek et al., 2010; Salcher et al., 2015). During 24 hours after sampling, fixed samples (1-10 ml) were filtered on 0.2 em pore size polycarbonate filters (Osmonic Inc., Livermore, CA or Millipore Corporation, Billerica, MA) and filters were stored at -20°C or immediately processed. The CARD-FISH protocol (Sekar et al., 2003) was slightly modified concerning the duration of the achromopeptidase treatment for some samples (20-45 min) and optimized for the newly developed probe LimAE-1435 by a stepwise increase of formamide concentrations in the hybridisation buffer (25% - 70%). Probes LimAE-1435 and R-BT065 (Šimek et al., 2001) were used for double hybridisation (Sekar et al., 2004) followed by amplification with fluorescein (probe LimAE-1435) and Alexa546 (probe R-BT065) labelled tyramides (Invitrogen Corporation, Carlsbad, CA), respectively, to distinguish lineages LimA and LimE (Fig. 2). The double hybridisation protocol was tested and optimized on isolates from LimA, LimE and LimC lineages, and their mixtures. The proportions of LimAE-1435 positive bacteria as well as that of double-hybridised bacteria (representatives of the LimE cluster) were determined by inspecting more than 500 DAPI stained cells with epifluorescence microscopy (Olympus BX-53F) using U-NWU, U-WB and U-WG optical filter sets. Alternatively, >1000 cells per sample were analysed by fully automated high-throughput microscopy (Zeder and Pernthaler, 2009) using Axioimager M1 (Carl Zeiss; optical filter sets: 01, 09 and 14) and the ACMEtool image analysis software (technobiology.ch). A quantification limit was set to 0.2% of DAPI stained cells. A separate hybridisation of R-BT cells and subsequent amplification with fluorescein labelled tyramides facilitated a quantification of this group by avoiding interference of autotrophic cells signals. Abundances of PnecC were assessed using a specific probe (Hahn et al., 2005).

Physiological profiling

Metabolic profiles of five strains of the LimA (Jir61, JirI-52, Rim8, Sp3 and *L. australis* MWH-BRAZ-DAM-2D^T) and three strains of the LimC lineage (Rim47, *L. parvus* II-B4^T, *L. planktonicus* II-D5^T) were assessed with PM1 microplates (BioLog, Hayward, USA) at 22°C in carbon-free artificial lake water medium amended with vitamins (Zotina et al., 2003). An absorption microplate reader (Spectra Max 190, Molecular Devices, USA) was used for monitoring growth at 600 nm with 15 min intervals during 4 days. The bacterial growth was evaluated as following: The minimum absorbance value was subtracted from the mean of 5-10 absorbance measurements during the stationary phase for each substrate and the obtained result was normalized to the negative control of evaluated strain.

Statistical analysis

RDA was conducted to determine the effect of biological and physicochemical parameters on relative and absolute abundances of LimA and R-BT in samples of Lake Zurich and Římov reservoir. A normal distribution of raw data was achieved by logarithmic transformation (log[x+1]) of explanatory variables and absolute abundances, and by arcsine transformation of relative abundances. Additionally, partitioning of variation of LimA and R-BT densities was used to access the explanatory potential of physicochemical vs. phytoplanktonic parameters in both ecosystems. For samples from highly dynamic freshwater ecosystems (Table S6), effects of environmental variables on absolute abundances of three groups of Betaproteobacteria (LimA, R-BT and PnecC) was analysed by CCA. Significant variables ($P<0.05$) were chosen according to their simple effects with 999 Monte Carlo permutations for all analyses using Canoco 5 software (www.canoco5.com). A Kruskal-Wallis one-way analysis of variance was used to test for significant differences between samples taken from different stations in the Římov reservoir and pairwise multiple comparisons were performed by Dunn's test using SigmaPlot version 12.5, (Systat Software, Inc., San Jose California USA, www.sigmaplot.com). A Mann-Whitney rank sum test was applied to verify differences between samples taken at stagnant water conditions and after flood events at the inflow of the Římov reservoir (station River). PCA using Pearson's correlation coefficients was conducted on data obtained from physiological profiling in XLSTAT 2014 (www.xlstat.com).

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Figure and table legends

Fig. 1

Phylogenetic reconstruction of *Limnohabitans* and related genera (Comamonadaceae). Maximum likelihood tree based on sequences of 23S rRNA genes (length approx. 2100 bp). Branches with bootstrap values below 30% were resolved to multifurcations and values above 40% are displayed for key nodes. The scale bar on the bottom corresponds to 1% sequence difference. Probes LimAE-1435 and R-BT065 are displayed on the left and groups of novel isolates separated by high bootstrap values are marked by Roman numerals.

Fig. 2

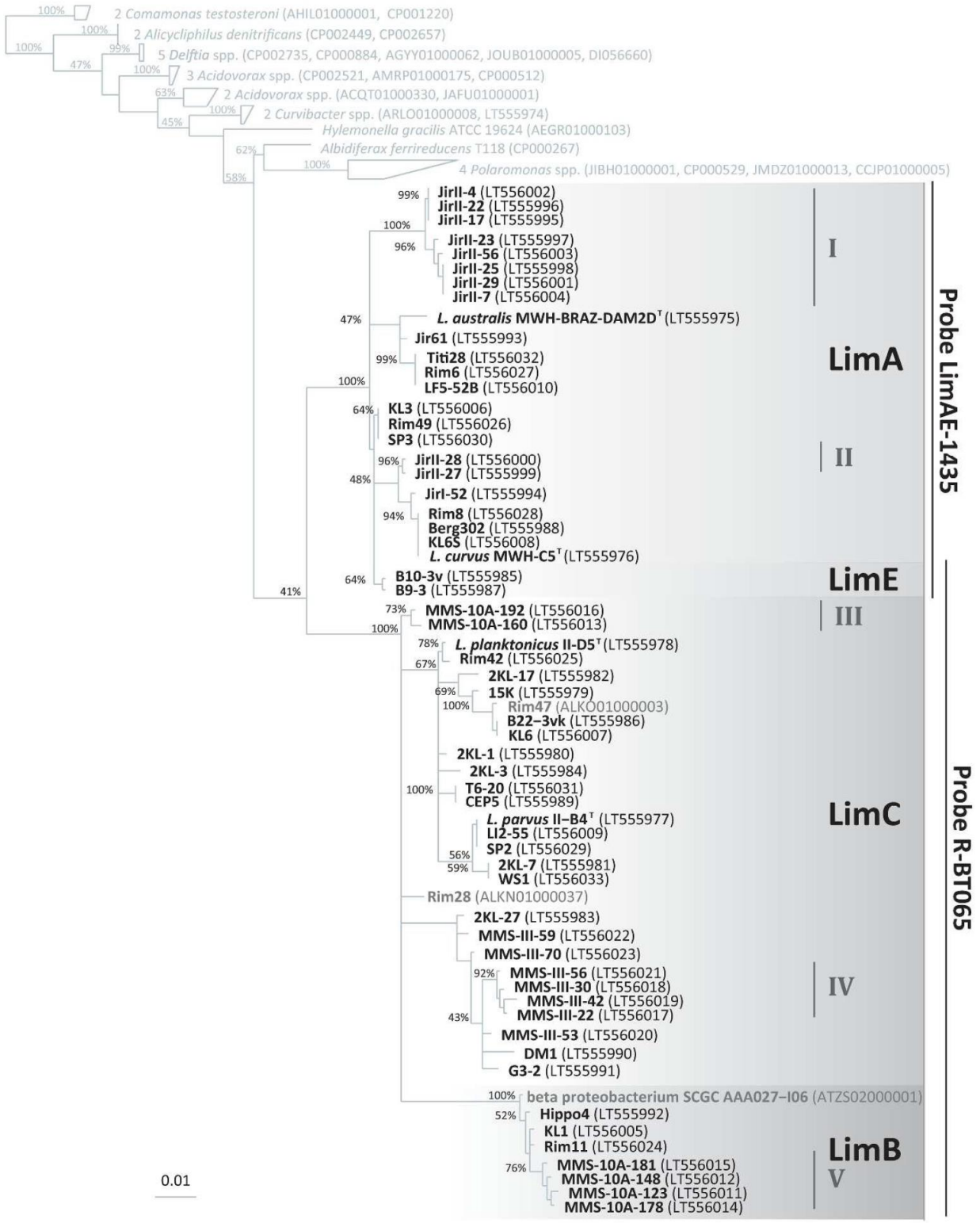
Double hybridisation with probes LimAE-1435 and R-BT065. A: UV excitation after DAPI staining (Carl Zeiss optical filter set 01; excitation BP 365/12, emission LP 397), B: blue excitation after staining with fluorescein (Carl Zeiss optical filter set 09; excitation BP 450-490, emission LP 515), C: green excitation after staining with Alexa546 (Carl Zeiss optical filter set 14; excitation BP 510-560, emission LP 590). Cells hybridised only with probe LimAE-1435 (LimA) are highlighted green, cells hybridized only with probe R-BT065 (LimBCD) are highlighted red, double hybridised cells (LimE) are highlighted orange.

Fig. 3

Abundances of LimA and R-BT groups in Lake Zurich during 2010-2012. (a) Average concentrations of diatom chlorophyll a in 0-20 m depths (20 depth layers each). (b) Multiannual averages of relative abundances of R-BT in nine depths layers (78 sampling dates). (c) Absolute abundances of R-BT (n=737). (d) Average concentrations of green algae (Chlorococcales) chlorophyll a in 0-20 m depths (20 depth layers each). (e) Multiannual averages of relative abundances of LimA in nine depths layers (78 sampling dates). (f) Absolute abundances of LimA (n=737). (g) Redundancy analysis of abundances of LimA and R-BT in epilimnion of Lake Zurich in context of significant physicochemical and biological parameters. Grey dashed lines represent variables with $0.05>P>0.01$; grey continuous lines represent variables with $P<0.01$; O_2, oxygen concentrations; Org N, organic nitrogen; P-Chl, chlorophyll a associated with *Planktothrix rubescens*; C-Chl, chlorophyll a associated

778 with Cryptophyta; D-Chl, chlorophyll a associated with diatoms; TPB, total
779 phytoplankton biomass; G-Chl, chlorophyll a associated with green algae
780 (Chlorococcales); DP, dissolved phosphorus; DOC, dissolved organic carbon; NH4,
781 ammonium concentrations; NO_3, nitrate concentrations; PO_4, phosphate
782 concentrations; R-BT and LimA, absolute abundances of these groups; R-BT% and
783 LimA%, relative abundances of these groups in percentages of DAPI stained cells.
784 (h) Results of variation partitioning. Physicochemical, group of physicochemical
785 parameters: concentrations of dissolved phosphorus, dissolved organic carbon,
786 ammonium, nitrate, phosphate, temperature, sample depth and conductivity;
787 Phytoplankton, group of phytoplankton related parameters: concentrations of
788 chlorophyll a associated with *Planktothrix rubescens*, Cryptophyta, diatoms and
789 green algae, and total phytoplankton biomass.
790
791 **Fig. 4**
792 Distribution of relative and absolute abundances of (a) LimA and (b) R-BT at three
793 sampling stations of the Římov reservoir during 2011-2013. Different letters
794 correspond to significant differences ($P<0.05$, Tukey test) between average values.
795 (c) RDA of R-BT and LimA abundances in context of significant physicochemical and
796 spatial and seasonal parameters. Grey dashed lines represent variables with
797 $P=0.05$; grey continuous lines represent variables with $P<0.05$; Chl A, total
798 chlorophyll *a* concentrations; Cy-Chl, cyanobacterial chlorophyll a concentrations;
799 DRP, dissolved reactive phosphorus; TP, total phosphorus; River, station of the
800 Římov reservoir; Spring and Summer, seasons; A370, absorbance measured at 370
801 nm excitation *in situ*; R-BT and LimA, absolute abundances of these groups; R-BT%
802 and LimA%, relative abundances of these groups in percentages of DAPI stained
803 cells.
804
805 **Fig. 5**
806 Abundances of LimA at station River of Římov reservoir in context of flow rate. (a)
807 Relative abundances of LimA and flow rate during 2011-2013. White circles
808 correspond to relative abundances of LimA at normal hydrological conditions, black
809 circles correspond to relative abundances of LimA at post flood conditions (up to 3
810 weeks after flood event (flow rate >17.8 m^3 s^{-1})). Grey areas represent periods of ice
811 coverage that were not sampled, and are shown compressed along the time-axis. (b)

812 Relative and absolute abundances of LimA at post flood and normal hydrological
813 conditions. Different letters correspond to significant differences (P<0.001, Mann-
814 Whitney rank sum test) between average values.
815
816 **Fig. 6**
817 Canonical correspondence analysis of absolute abundances of LimA, R-BT and
818 PnecC, and environmental variables from 28 samples from 22 highly dynamic
819 aquatic ecosystems. Grey dashed lines represent variables with 0.05>P>0.01; grey
820 continuous lines represent variables with P<0.01; SUVA, $SUVA_{254}$ - ratio between
821 specific UV absorbance measured at 254 nm and DOC concentration; Chl A, total
822 chlorophyll *a* concentrations; A250, absorbance measured at 250 nm excitation.
823
824 **Fig. 7**
825 Physiological profiling of LimA and LimC strains tested on 95 substrates of BioLog
826 PM1 plates. (a) Numbers of substrates belonging to different organic compounds
827 classes utilized by different *Limnohabitans* strains. (b). Principal component analysis
828 of growth efficiencies of LimA strains (Rim8; *L. australis*, strain MWH-BRAZ-DAM-
829 $2D^T$; SP3; JirI-52 and Jir61) and LimC strains (*L. parvus*, strain II-$B4^T$; L.
830 planktonicus, strain II-$D5^T$ and Rim47).
831
832 **Table 1.**
833 Ranges of LimA and R-BT abundances in different environments. n, number of
834 analysed samples; %, percentages of DAPI stained cells; NA, not available.



835

836

837 Figure 1

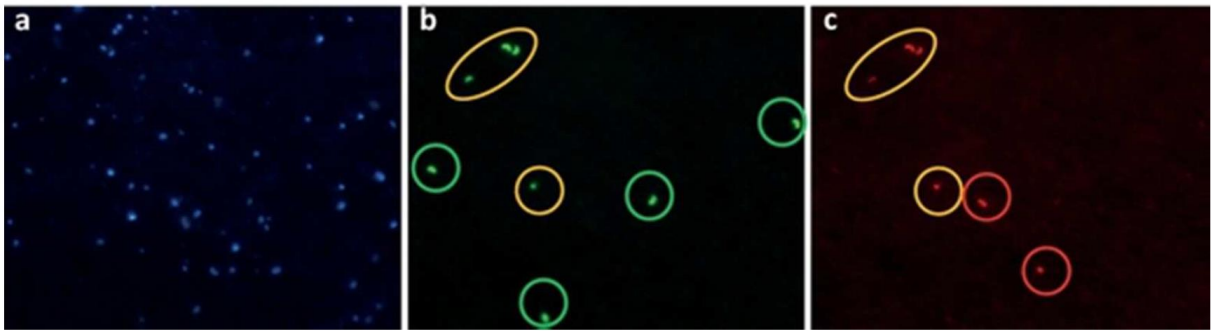
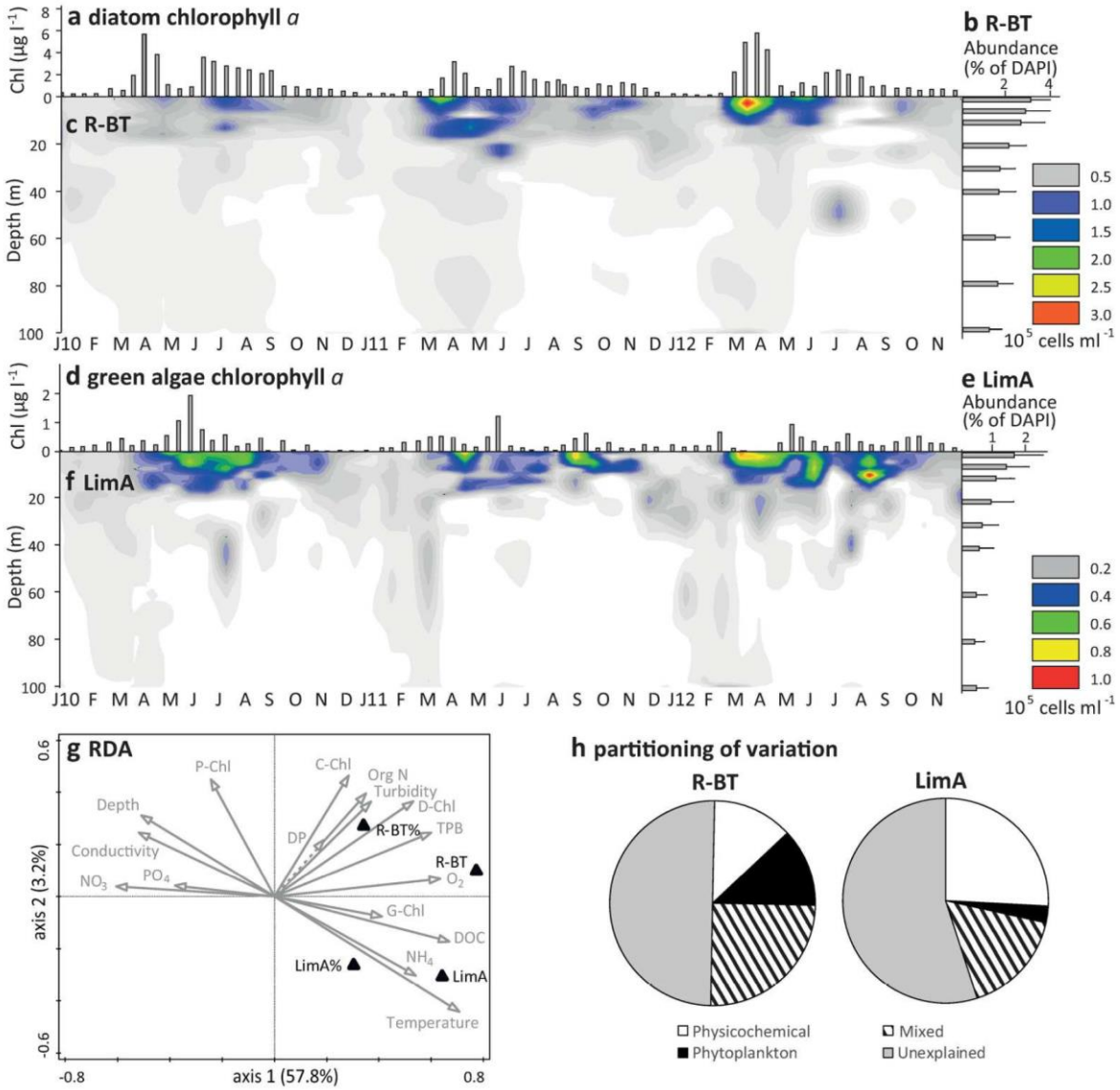


Figure 2



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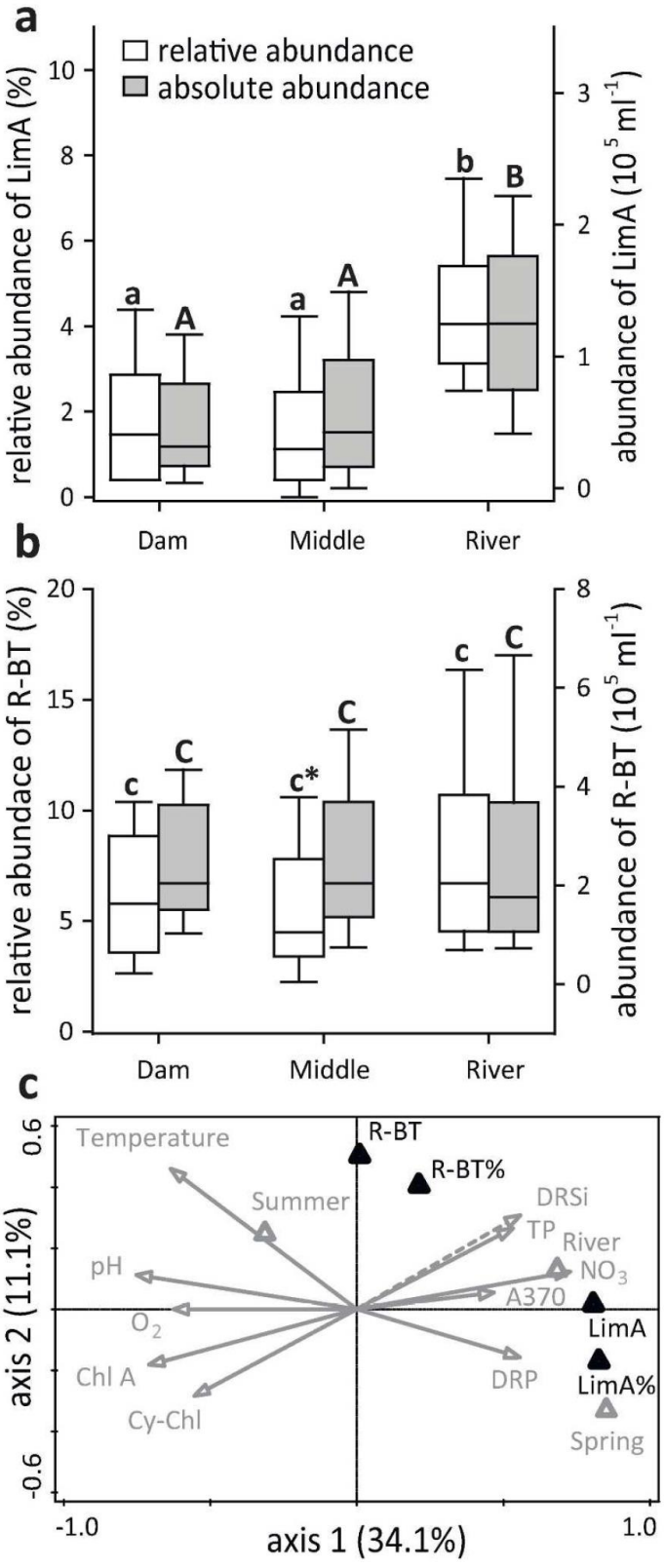
843 Figure 3



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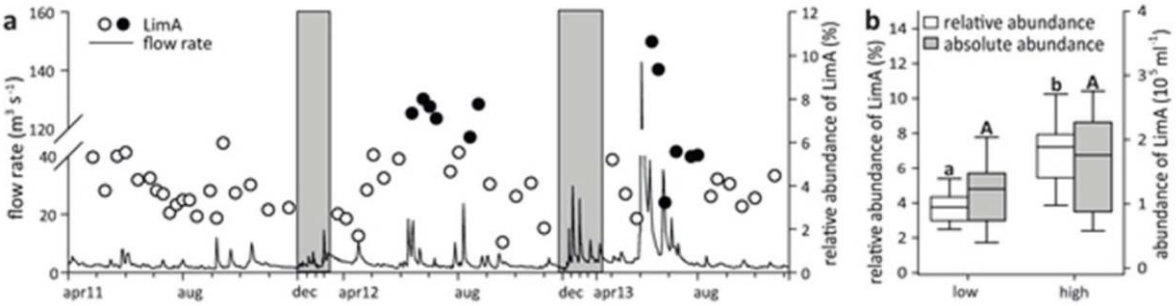
845

846 Figure 4



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848

849 Figure 5

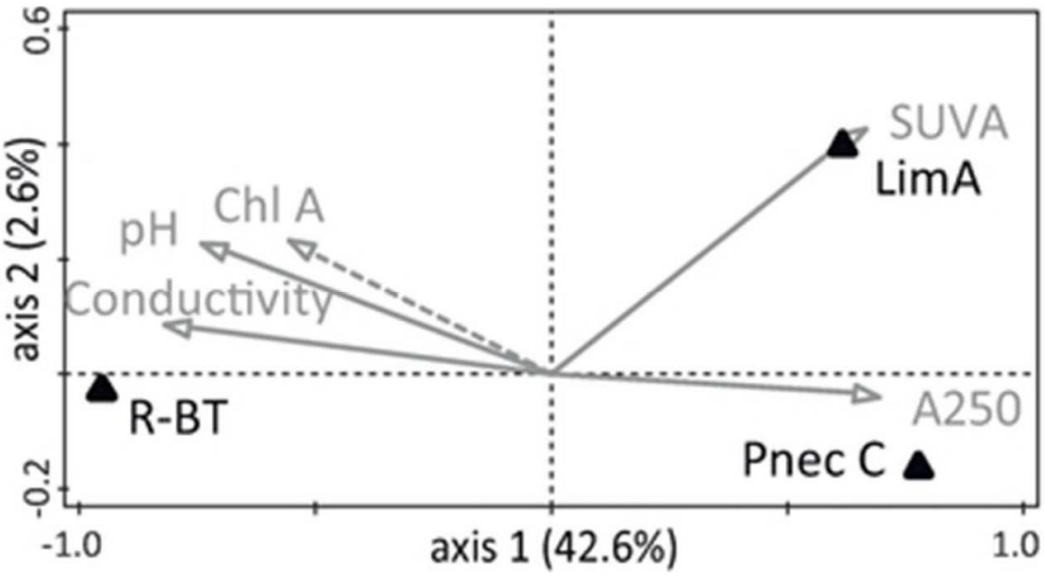
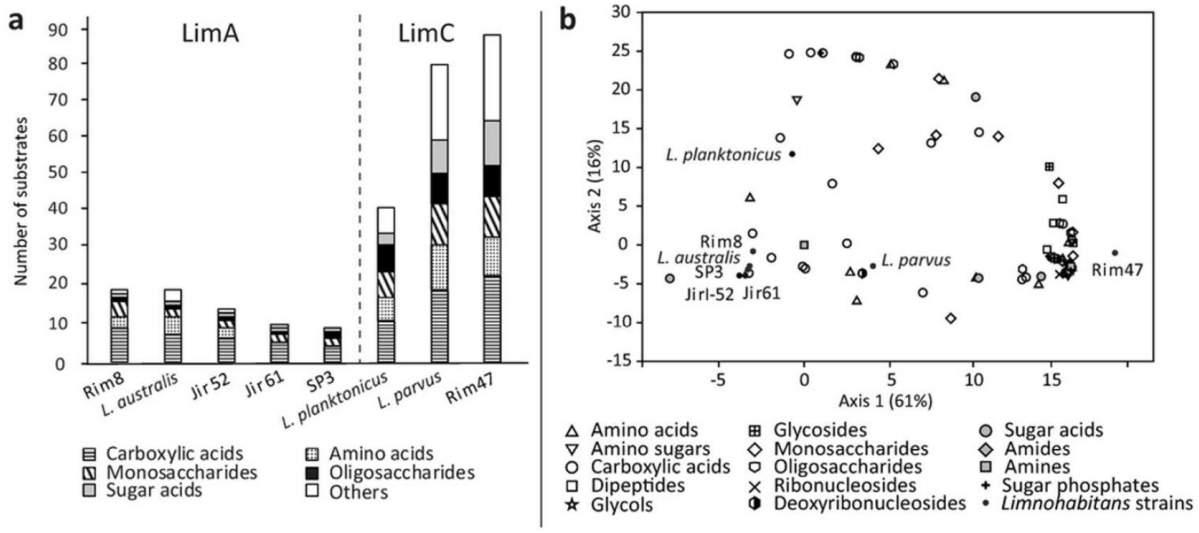

0.6
-0.2
axis 2 (2.6%)
SUVA
LimA
Chl A
pH
Conductivity
R-BT
A250
Pnec C
-1.0
axis 1 (42.6%)
1.0

Figure 6



853

854

855 Figure 7

856 Table 1.

857

	n	LimA %	LimA 10^5 cells ml^{-1}	R-BT %	R-BT 10^5 cells ml^{-1}	LimE %	LimE 10^5 cells ml^{-1}
Lake Zurich	737	0-5.4	0-1.1	0-6.7	0-2.7	0-1.5	0-0.4
Římov reservoir	168	0-14.9	0-3.6	1.2-19.4	0.4-8.9	0-1.9	0-0.5
Other lakes	50	0-6.1	NA	0-14.3	NA	0-14.3	NA
Ponds	29	0-18.6	0-14.8	0-28.8	0-12.0	0-2.7	0-0.6
Springs	2	8.7-17.3	NA	0	0	0	0
Peat bogs	4	0	0	0	0	0	0
Algal cultures	6	0	0	9.1-26.2	NA	0	0
Cultures of macrophytes	3	3.9-45.5	0.7-9.0	0-1.7	0-0.4	0	0